The Induction of the Enzymes of Naphthalene Metabolism in *Pseudomonads* by Salicylate and 2-Aminobenzoate

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INTRODUCTION

Naphthalene oxygenase is induced in certain *Pseudomonas* species by growth on salicylate, and may be induced gratuitously by 2-aminobenzoate or by 2-hydroxybenzyl alcohol in cultures growing on succinate (Shamsuzzaman & Barnesley, 1974*a*). The induction of a number of enzymes responsible for the conversion of naphthalene to non-aromatic products (Davies & Evans, 1964) has now been measured in *Pseudomonas* NCIB9816 and *Pseudomonas putida* (strains ppG7 and ATCC17483) grown on succinate, or an inducer of naphthalene oxygenase. This paper reports these observations and discusses the regulation which may be involved.

METHODS

Organisms and culture media. *Pseudomonas* NCIB9816 was obtained from the National Collection of Industrial Bacteria, and *Pseudomonas putida* (strain ATCC17483) was obtained from the American Type Culture Collection. Strain ppG7 of *Pseudomonas putida* (Dunn & Gunsalus, 1973), a gift from Dr I. C. Gunsalus, was derived originally from the collection of Stanier, Palleroni & Doudoroff (1966) (strain 111), and was deposited by the latter authors in the American Type Culture Collection as ATCC17485. Samples of ATCC17485 obtained directly from the American Type Culture Collection could not be induced to oxidize naphthalene, and nor could a copy of strain 111 kindly provided by Dr M. Doudoroff.

Carbon sources, 2-aminobenzoate, and the basal mineral medium used for cultures of NCIB9816 and ATCC17485 were prepared as described by Shamsuzzaman & Barnesley (1974*a*). *Pseudomonas putida* tended to clump during the early phase of growth in this mineral medium, and the effect was particularly pronounced with ppG7. Consequently, the mineral medium of Jacobson, Bartholomaeus & Gunsalus (1966) was used for the latter organism.

Culture of cells and preparation of cell extracts. Cultures (400 ml) were grown at 25 °C in 11 flasks shaken at 150/osc./min in a New Brunswick Scientific G76 shaker. Solutions of inducers or other carbon compounds, sterilized by filtration, were added during the early exponential phase of growth (apparent extinction about 0.2 at 600 nm measured with 1 cm cuvettes in a Perkin Elmer-Coleman 124 spectrophotometer), and after the population had doubled, about twice the cells were collected by centrifugation at 6000 *g* for 20 min at 2 °C. The bacterial pellet was washed in ice-cold 50 mM-potassium dihydrogen phosphate-NaOH buffer pH 7.0 and resuspended (0.1 g wet wt/ml) in phosphate buffer. Part of the suspension was disrupted with an ultrasonic oscillator (Model 185, Heat Systems-Ultrasonics, Inc., Plain View, New York, U.S.A.) at a nominal power of 60 W. A portion (3 to 4 ml) initially at 0 °C was disrupted for four 0.5 min periods. Each period of disruption was

followed by 1 min during which the disrupted cells and oscillator probe were cooled in ice water. The preparation was then centrifuged at 40000 g for 1 h at 4 °C, and the supernatant was stored on ice.

Determination of naphthalene oxygenase. The spectrophotometric method of Shamsuzzaman & Barnsley (1974*a*) using whole cells was employed. The results were not corrected for salicylate accumulation and therefore represent minimal values.

Determination of salicylate hydroxylase (EC. 1.14.13.1). Method 1. A procedure similar to that of Yamamoto *et al.* (1965) was used. To 2.45 ml phosphate buffer pH 7.0 at 25 °C were added 0.1 ml FAD (0.3 mM), 0.05 ml cell extract, 0.2 ml NADH (1.5 mM) and 0.2 ml sodium salicylate (1.5 mM), and the rate of decrease in extinction at 340 nm was measured. The rate was corrected for the rate of oxidation of NADH which occurred independently of added salicylate, and enzymic activity was calculated using an extinction coefficient of $6.22 \text{ mm}^{-1} \text{ cm}^{-1}$. The concentration of FAD used in this determination (10 μM) is very much higher than the K_m (0.07 μM) reported by Yamamoto *et al.* (1965) for the purified enzyme from a *Pseudomonas* species. Though added FAD was not always necessary to give the maximum rate of reaction with extracts of NCIB9816, it was invariably required with extracts of PpG7. Extracts of ATCC17483 appeared to contain no enzymic activity when assayed by this method, and so the following polarographic determination was used.

Method 2. To 1.85 ml of phosphate buffer pH 7.0 at 25 °C were added 0.033 ml FAD (0.6 mM), 0.033 ml NADH (6.0 mM) and 0.05 ml of cell extract. The salicylate-independent rate of oxygen uptake was measured for 2 min, and then 0.033 ml sodium salicylate (6.0 mM) was added. The rate of oxygen uptake was measured, corrected for the salicylate-independent uptake, and the enzymic activity was calculated assuming an initial O_2 concentration of 0.25 mM.

Determination of other enzymes. Salicylaldehyde dehydrogenase and 1,2-dihydroxynaphthalene oxygenase (Shamsuzzaman & Barnsley, 1974*b*), catechol 1,2-dioxygenase (EC. 1.13.11.1) (Hegeman, 1966) and catechol 2,3-dioxygenase (EC. 1.13.11.2) (Feist & Hegeman, 1969) were determined by published methods. Catechol 2,3-dioxygenase was determined immediately a supernatant was obtained.

RESULTS AND DISCUSSION

Table 1 gives the levels of enzymes in whole cells and extracts of NCIB9816, ATCC17483 and PpG7, respectively, under various conditions of growth. In all cases the presence of 2-aminobenzoate or salicylate caused the induction of naphthalene oxygenase, 1,2-dihydroxynaphthalene oxygenase, salicylaldehyde dehydrogenase, and salicylate hydroxylase. In ATCC17483 and PpG7, catechol 2,3-dioxygenase was also induced. 2-Aminobenzoate must have induced gratuitously for its concentration in the medium, determined as described by Shamsuzzaman & Barnsley (1974*a*), remained unchanged during the growth of the culture to the stationary phase. Gratuitous induction of salicylate hydroxylase in NCIB9816 was not noted earlier because extracts were not fortified with FAD.

The induction of all these enzymes by a gratuitous inducer also suggests that they may be co-ordinately induced, but confirmation of this in terms of a constant proportionality of enzyme levels and a common time course of induction (compare Ornston, 1966) is lacking due to technical difficulties in the measurement of the activities of enzymes in complex mixtures.

The possibility of the co-ordinate regulation of the enzymes which convert naphthalene to 2-hydroxymuconic semialdehyde is of particular interest as Dunn & Gunsalus (1973)

Table 1. *The specific activities of certain enzymes in Pseudomonas species grown in the presence of different compounds*

Cells were grown on the given carbon source and harvested in the exponential phase, or grown on succinate in which case the second compound was added early in the exponential phase (E_{500}^{600} about 0.2) and cells were harvested after doubling twice. Concentrations are given in parentheses. Enzymic activities were determined with whole cells (naphthalene oxygenase) and extracts (other enzymes), and calculated specific activities are based on a unit weight of protein in whole cells or extract as appropriate. Method 2 was used for salicylate hydroxylases only in ATCC17483; method 1 was used for all other organisms.

Initial carbon source (mM)	Inducer (mM)	Specific activity (μmol/min/mg protein)				
		Naphthalene oxygenase	Dihydroxy-naphthalene oxygenase	Salicylaldehyde dehydrogenase	Salicylate hydroxylase	Catechol 1,2-dioxygenase
<i>Pseudomonas</i> sp. NCIB9816						
Succinate (8.5)	—	0.007	0.05	0.06	0.001	0.003
Naphthalene (0.1 %)	—	0.355	3.20	1.31	0.245	0.284
Salicylate (3.5)	—	0.300	3.95	1.36	0.308	0.288
Succinate (8.5)	Salicylate (3.5)	0.250	2.61	0.56	0.358	0.138
Succinate (8.5)	2-Aminobenzoate (0.35)	0.240	2.39	0.60	0.296	0.007
Succinate (8.5)	Catechol (3.0)	0.006	0.03	0.03	0.002	0.275
<i>Pseudomonas putida</i> ATCC17483						
Succinate (8.5)	—	0.007	0.00	0.01	0.005	0.001
Naphthalene (0.1 %)	—	0.323	1.02	1.17	0.119	0.023
Salicylate (3.5)	—	0.291	1.62	1.33	0.082	0.018
Succinate (8.5)	Salicylate (3.5)	0.051	0.86	0.21	0.080	0.010
Succinate (8.5)	2-Aminobenzoate (0.35)	0.072	0.692	0.57	0.105	0.019
Succinate (8.5)	Catechol (3.0)	0.002	0.001	0.00	0.001	0.004
<i>Pseudomonas putida</i> ppG7						
Succinate (8.5)	—	0.003	0.03	0.22	0.001	0.001
Naphthalene (0.1 %)	—	0.510	0.43	1.80	0.238	0.325
Salicylate (3.5)	—	0.520	0.30	1.76	0.286	0.401
Succinate (8.5)	Salicylate (3.5)	0.140	0.26	1.02	0.190	0.220
Succinate (8.5)	2-Aminobenzoate (0.35)	0.163	0.27	1.63	0.314	0.010
Succinate (8.5)	Catechol (3.0)	0.001	0.00	0.10	0.002	0.376
Succinate (8.5)	Catechol (3.0)	0.001	0.00	0.10	0.002	0.002

have demonstrated that the genes which specify the enzymes for these reactions in ppG7 are plasmid borne. Enzymes further along the degradative pathway have not been examined, but Chakrabarty (1972) showed that in certain other pseudomonads the genes for salicylate degradation through catechol and the meta pathway are also plasmid borne. An extension of the present observations and an examination of the possible homology of the naphthalene and salicylate plasmids would be of the greatest interest.

Note added in proof. Dr P. A. Williams kindly informed the author (personal communication) of his use of 10% acetone to stabilize catechol 2,3-dioxygenase in extracts of NCIB9816, but I have not been able to confirm the induction of this enzyme.

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REFERENCES

- CHAKRABARTY, A. M. (1972). Genetic basis of the biodegradation of salicylate in *Pseudomonas*. *Journal of Bacteriology* **112**, 815-823.
- DAVIES, J. I. & EVANS, W. C. (1964). Oxidative metabolism of naphthalene by soil pseudomonads. The ring fission mechanism. *Biochemical Journal* **91**, 251-261.
- DUNN, N. W. & GUNSALUS, I. C. (1973). Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. *Journal of Bacteriology* **114**, 974-979.
- FEIST, C. F. & HEGEMAN, G. D. (1969). Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. *Journal of Bacteriology* **100**, 869-877.
- HEGEMAN, G. D. (1966). Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. *Journal of Bacteriology* **91**, 1140-1154.
- JACOBSON, L. A., BARTHOLOMAUS R. C. & GUNSALUS, I. C. (1966). Repression of malic enzyme by acetate in *Pseudomonas*. *Biochemical and Biophysical Research Communications* **24**, 955-960.
- ORNSTON, L. N. (1966). The conversion of catechol and protocatechuate to β -keto adipate by *Pseudomonas putida*. *Journal of Biological Chemistry* **241**, 3800-3810.
- SHAMSUZZAMAN, K. M. & BARNSLEY, E. A. (1974*a*). The regulation of naphthalene oxygenase in pseudomonads. *Journal of General Microbiology* **83**, 165-170.
- SHAMSUZZAMAN, K. M. & BARNSLEY, E. A. (1974*b*). The regulation of naphthalene metabolism in pseudomonads. *Biochemical and Biophysical Research Communications* **60**, 582-589.
- STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**, 159-271.
- YAMAMOTO, S., KATAGIRI, H., MAENO, H. & HAYAISHI, O. (1965). Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. I. Purification and general properties. *Journal of Biological Chemistry* **240**, 3408-3413.